

Lipase Production from *Bacillus subtilis* using various Agricultural waste

*Naaz Abbas¹, Javeria Javed², Zainab Abbas³, Shahnaz Choudry, Sakhawat Ali¹

¹Food & Biotechnology Research Center, Pakistan Council of Scientific & Industrial Research (PCSIR) Laboratories Complex, Ferozpur Road Lahore, Pakistan.

²Department of Biotechnology, Kinnaird College for Women 93-Jail Road Lahore
China University of Geosciences, Wuhan, China.

Abstract— Lipases was produced by *Bacillus subtilis* PCSIR NL-38 strain and rape seed oil cake as substrate. Surface fermentation of minimal media in 250ml conical flask under static conditions gave 12.81 U/ml of lipases at 40°C for 48 hours. Lipase activity was monitored titrimetrically. Optimization of physicochemical parameters indicated that PCSIR NL-38 showed maximum lipase production at pH 7 with NH_4NO_3 as inorganic nitrogen source, glucose as carbon source, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ as salt, with 7% inoculum size and 96 hours of incubation.

Keywords— Lipase, *Bacillus subtilis*, Surface Fermentation, agricultural wastes, optimization.

I. INTRODUCTION

Production of enzymes through microorganisms is the major prospect of biotechnology. Lipases (glycerol ester hydrolases, EC 3.1.1.3) catalyse esterification and hydrolysis of fats into glycerol and fatty acids, depending upon the availability of water [1]. They also take part in interesterification, transesterification and acidolysis [2]. Due to their unique characteristics it remains attractive towards various microbiologists to optimize the conditions for obtaining it on large scale. Conventionally lipases were obtained from animals and plants but due to their limited production and post-translational modifications. Microbial source is preferred because of their convenient production, safer and can be produced in bulk amount at subsidiary low cost [3].

While catalyzing the reaction lipases do not need any cofactor and they also do not catalyse side reactions which make them more specific in chemical reactions. Such versatile properties allow them to be use in different fields like food, chemical, oleochemical and pharmaceutical industries [4], [5]. In hydrolysis and modification of oil and fat the hydrolytic behavior of lipases is applied which help to hydrolyse ester bonds [5], [6]. They are applied as an additive in formulation of detergent for removing fatty stains as they hydrolyze ester bonds in the presence of excess water [7]. Lipases are used in fur processing as they helped to remove fats from animal fur and make it more convenient and environment friendly as they substitute

hazardous chemicals which increase pollution [8]. In some bakery products lipases are used for enhancing aromatic properties and taste of some commercially important products like milk, cheese, butter, and yogurt and enhance their shelf life and nutritious value [9]. The present research was undertaken to optimize the production of extracellular lipases from *Bacillus subtilis* PCSIR NL-38

II. MATERIAL AND METHODS

Revival of isolates

Ten bacterial isolates were obtained from Molecular biology lab at FBRC in PCSIR and revived on Luria Bertani agar plates at 30°C for 24 hours.

Screening of lipolytic bacterial isolate

Selection of lipolytic strains was done by plating on tributyrin agar plates containing: 0.5% peptone, 0.3% yeast extract, 1.2% agar and 2% tributyrin. Further selection of best lipase producer was done by adding fermented broth on Tween-80 agar plates containing 0.1% calcium chloride, 0.01% phenol red, 1% Tween-80 and 2% agar. Selected strain was stored on LB agar plates at 4°C. Morphological and biochemical characterization was done according to Bergey's Manual of determinative Bacteriology.

Effect of various agricultural wastes

Crushed rape seed oil cake, cotton seed cake, crushed almond oil cake, wheat bran, molasses and sugar cane bagasse was used as substrates. They were obtained from a local market of Lahore, Pakistan.

Preparation of inoculum

Vegetative inoculum was prepared by adding loopful of bacterial cells PCSIR NL-38 in 250ml conical flask containing 50 ml of minimal media (g/L): KH_2PO_4 3.0, Na_2HPO_4 6.0, NaCl 5.0, NH_4Cl 2.0 and MgSO_4 0.1. Flask was then placed in incubator for 24 hours at 32°C.

Preparation of fermentation medium

Fermentation medium was prepared by soaking 5 g of desired substrate into above mentioned minimal medium and autoclaved at 15 psi for 15 minutes.

Surface fermentation

The flasks containing fermentation media were inoculated with 3% of prepared inoculum and incubate at 32°C under static condition for 48 hours.

Extraction of Lipases

Fermented lipase was then extracted by adding 0.05M potassium phosphate buffer (pH 8) and placed on orbital shaker at speed 1 for one hour. The samples were then filtered with the linen cloth and centrifuge 1 ml of supernatant at 5000 rpm for 10 minutes at 4°C.

Titrimetric assay for lipase

Lipase activity was measured by adding 0.5ml of supernatant in 5ml of olive oil substrate (10% v/v olive oil in 10% w/v gum arabic). 1 ml of 0.6% CaCl₂ was added along with 2.5 ml of 1M phosphate buffer (pH 7). It was then placed on orbital shaker for 1 hour. To stop the biochemical reaction 10 ml of 1:1 acetone and ethanol was added. Liberated fatty acids then titrated against 0.1N NaOH by using 0.1% phenolphthalein as an indicator. Light pink was its end point [10].

Lipase unit is defined as “in the presence of definite assay conditions number of fatty acids released per ml due to enzyme action in one hour of incubation”.

Optimization of physicochemical parameters

Different physicochemical parameters were selected and optimized for maximum lipase production. These parameters include substrate selection, fermentation type, moisture content, inoculum size, pH of medium, carbon and nitrogen source, salts, incubation time and temperature.

III. RESULTS AND DISCUSSION

Screening of lipolytic bacterial isolate

Out of ten isolates six showed clear hydrolytic zone due to degradation of tributyrin around bacterial streak, indicating positive lipolytic activity. On tween-80 agar plates big hydrolytic diameter with change in color from red to yellow was observed by PCSIR NL-38 indicating it best lipase producer hence, selected for further study.

Morphological and biochemical characterization

Morphological characterization of PCSIR NL-38 (fig.1) illustrated that its colony have orange pigmented with opaque appearance, round configuration, smooth margins, slightly raised elevation, rod shaped, strict aerobic and non-motile. PCSIR NL-38 showed positive result for gram staining, spore staining, Methyl Red-Voges Proskauer test, starch hydrolysis, casein hydrolysis, catalase test, and citrate utilization, growth in 6.5% NaCl and at 55 ° C. Whereas, it showed negative result for oxidase, H₂S production and gas production in xylose, sucrose, lactose

and arabinose. Therefore, it was identified as *Bacillus subtilis*.

Effect of agricultural wastes as substrate on lipase production

Among all agricultural wastes shown in table 1, surface fermentation of rape seed oil cake gave maximum U/ml of lipases these results are in contrast to results given by Sekhon *et al.* [11] using neem seed cake.

Table.1: Effect of substrates in solid state and surface fermentation

Sr . #	Substrate	Lipase Activity in SSF (U/ml)	Lipase Activity in SRF (U/ml)
1	Cotton seed cake	0	0
2	Rape seed oil cake	12	12.81
3	Almond oil cake	0	2.02
4	Wheat bran	7	3.6
5	Bagasse	0	0.69

Effect of moisture content of substrate on lipase production

Results given in figure 2 illustrated that maximum units of lipase was achieved with 1:5 (substrate: moisture content) moisture content at 32°C for 48 hours.

Effect of inoculum size on lipase production

Optimum inoculum size for lipase production shown in figure 3 was 7% with respect to media. Previously 2.5% and 3.0% inoculum size was reported optimum by Begam *et al.* [12] and Iftikhar *et al.* [5] for solid state fermentation. 7% inoculum size is required because of adopted fermentation type, as no physical agitation was provided during fermentation.

Effect of pH of media on lipase production

Maximum enzyme units were obtained with pH 7. Results shown in figure 4 are parallel within pH range 6.5-8.0 reported by Sekhon *et al.* [11] but in contrast to results obtained at pH 6 and 9 by Begam *et al.* [12] and Padmapriya *et al.* [13].

Effect of different C and N source on lipase production

Comparing monosaccharide, disaccharide and polysaccharide, results shown in figure 5 indicated that 0.2% glucose was best carbon source for enhancing lipase production may be due to its easy availability to PCSIR NL-38. These results were parallel to previously observed by Iftikhar *et al.* [4] however, in contrast to results given by Ertugrul *et al.* [14]. Results shown in fig. 6 indicated

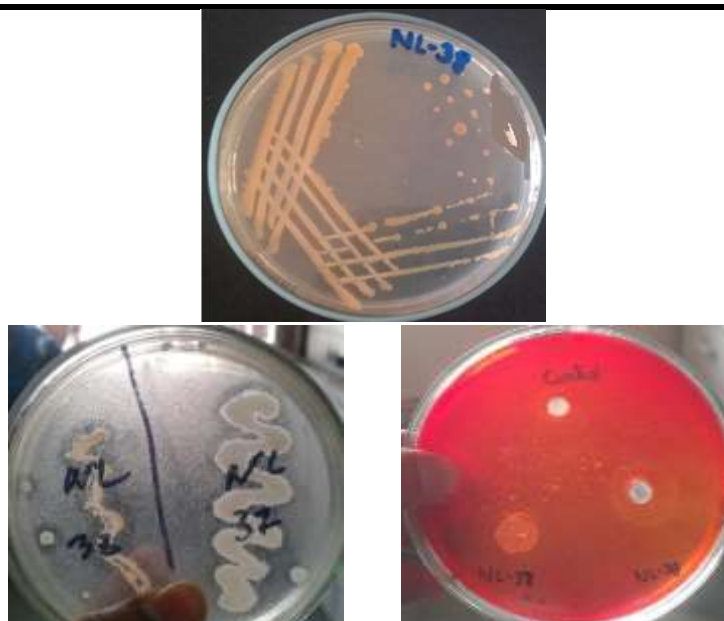


Fig. 1: (a) Colony morphology of PCSIR NL-38, (b) Clear zone around bacterial streaks NL-37 and PCSIR NL-38 on tributyrin agar plate and (c) Detection of lipolytic activity for the formation of zone with change in color around bacterial isolates NL-37 and PCSIR NL-38 on Tween 80 agar plates with 0.01% phenol red.

that ammonium nitrate as inorganic nitrogen source and peptone as organic nitrogen source gave maximum units of lipase. These results are better than results given by Kumar *et al.* [15].

Effect of different salts on lipase production

Results shown in fig. 7 indicated that ferrous sulphate instead of magnesium sulphate enhanced lipase production up to 2.5 fold.

Effect of temperature on lipase production

The reported temperature 40 °C in fig. 8 was in contrast to earlier reported temperature by Begam *et al.* [12] 25° C, Ertugrul *et al.* 14 30° C and Kumar *et al.* [15] 55° C as optimum for lipase production.

Effect of incubation time on lipase production

Fig. 9 revealed that maximum units of lipase were achieved by incubating for 96 hours at 32°C under static conditions. These results were in contrast to earlier reported incubation time of 48 hours by Sekhon *et al.* [11], Ertugrul *et al.* [14] and Padmapriya *et al.* [13] as optimum for lipase production.

IV. CONCLUSION

The screening and identification of lipolytic bacteria indicated that, *Bacillus subtilis* PCSIR NL-38 gave maximum lipase activity. It is concluded that surface fermentation of crushed rape seed oil cake gave 12.81U/ml of lipase under optimized conditions. The optimum values of incubation time, temperature and pH for the maximum lipase production was 48 hours, 40°C and 7. 7% inoculum size, 0.2% glucose (carbon source), and 0.2% peptone (organic nitrogen source) gave the

maximum enzyme production. Whereas, inorganic nitrogen source ammonium nitrate (0.2%) can also be used in place peptone for enhanced enzyme activity.

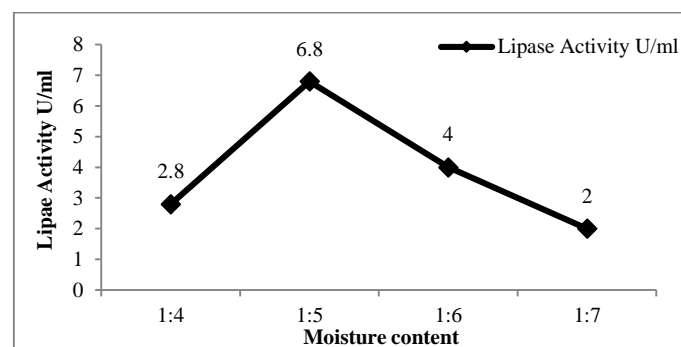


Fig. 2: Effect of moisture content on PCSIR NL-38

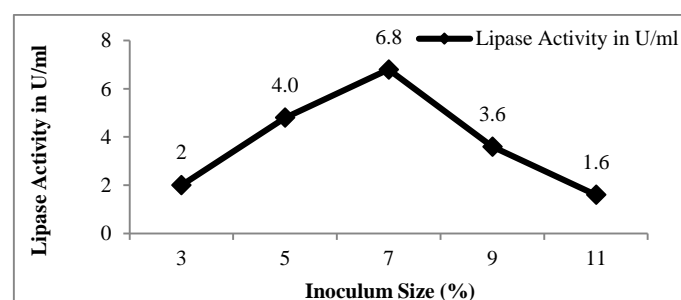


Fig. 3: Effect of inoculum size on PCSIR NL-38.

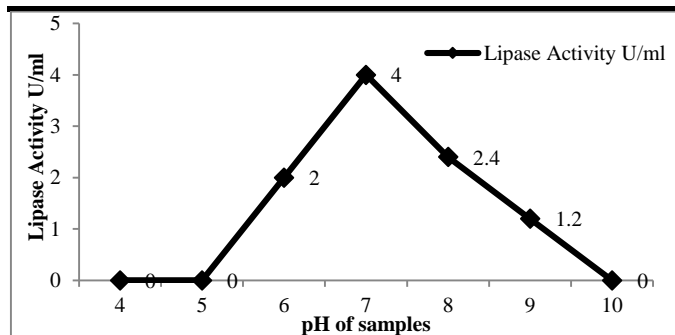


Fig. 4: Effect of pH for lipase production

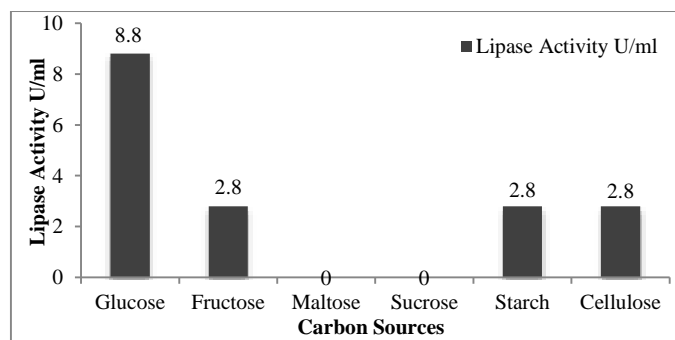


Fig. 5: Effect of carbon sources on lipase production

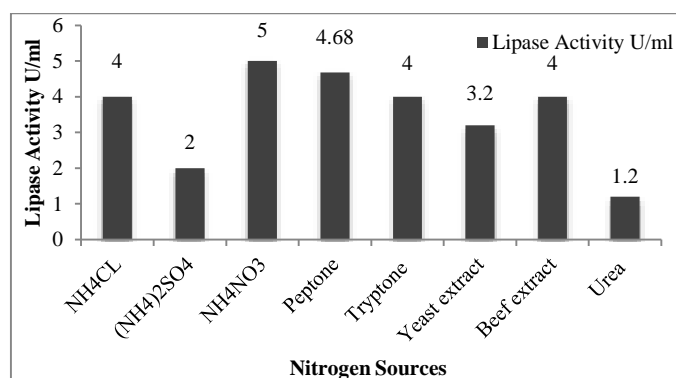


Fig. 6: Effect of nitrogen sources on lipase production

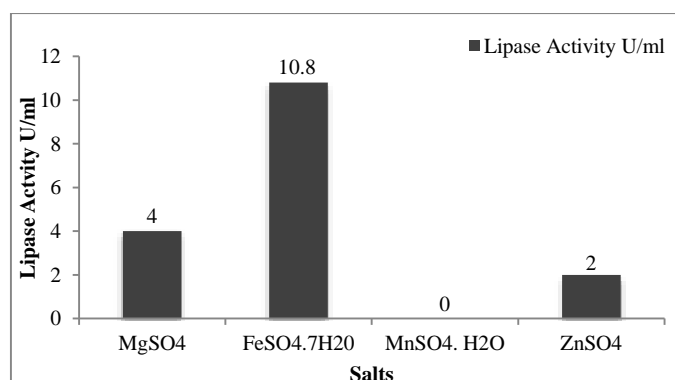


Fig. 7: Effect of different salts on lipase production

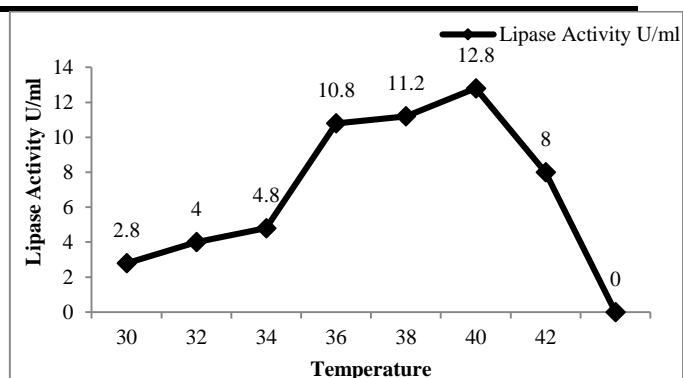


Fig. 8: Effect of temperature on lipase production

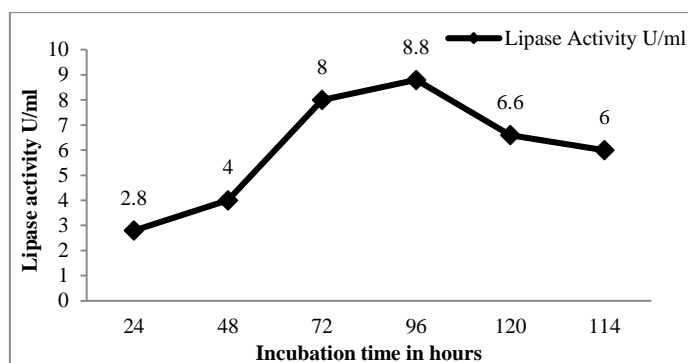


Fig. 9: Effect of incubation time on lipase production

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